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VOLATILES PRODUCTION AND ATTRACTIVENESS TO THE MEXICAN FRUIT FLY OF *Enterobacter agglomerans* ISOLATED FROM APPLE MAGGOT AND MEXICAN FRUIT FLIES

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Abstract—We investigated two strains of uricase (+) *Enterobacter agglomerans*, one isolated from the apple maggot fly (AMF) and one from the Mexican fruit fly (MFF), for 1) attractiveness to MFF, and 2) production of attractive chemicals. Regarding chemicals demonstrated attractive to the MFF, the MFF bacterial strain produced more 2,5-dimethylpyrazine, 2-phenylethanol, and indole than the AMF strain, whereas the AMF, but not the MFF strain, produced 3-hydroxybutanone. Cell types that predominated in plated subcultures varied from batch to batch resulting in variation in volatiles production, especially by the AMF strain where indole was sometimes a major component of the odor and at other times not detectable. Despite the greater production of attractive chemicals by the MFF strain, the AMF strain was consistently more attractive and the MFF strain was not different from uninoculated control plates. Statistical analyses indicated negative correlations of attractiveness with production of indole, 2,5-dimethylpyrazine, and 2-phenylethanol, and positive correlation with 3-hydroxybutanone. Results support previous findings with the Mexican fruit fly that showed combinations of attractive chemicals sometimes are not attractive.

Key Words—Attractants, Diptera, Tephritidae, *Anastrepha ludens*, ammonia, 3-methylbutanol, 3-hydroxybutanone, indole, solid phase microextraction (SPME).

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INTRODUCTION

During the past 20 years, numerous bacteria associated with fruit flies of the family Tephritidae have been identified (Fitt and O'Brien, 1985; Drew and Lloyd, 1989; Howard, 1989; Jang and Nishijima, 1990; MacCollom et al., 1992; Martinez et al., 1994; Kuzina et al., 2001; Marchini et al., 2002). In many cases, bacteria have been demonstrated to be attractive to the flies from which they were isolated (Jang and Nishijima, 1990; Robacker et al., 1991; MacCollom et al., 1992; Martinez et al., 1994). Various roles of these bacteria in the natural history of the flies have been proposed, including one as obligate symbiotes that flies transmit through various life stages (Petri, 1910; Allen and Riker, 1932; Hagen, 1966), facultative symbiotes that may convert unusable nitrogenous compounds into usable nitrogen (Lauzon et al., 2000), accidental symbiotes that are picked up by the flies while feeding (Huston, 1972; Howard, 1989), a source of protein (Drew et al., 1983; Drew and Lloyd, 1989), agents that detoxify unwanted components in food (Boush and Matsumura, 1967), or odor-producing indicators of proteinaceous foodstuffs to which flies are attracted (Robacker and Moreno, 1995). Bacteria could serve any one or several of these roles given different fruit fly species, bacteria species, and environmental and physiological circumstances.

A bacterium that has been closely tied to the natural history of the apple maggot fly (AMF), *Rhagoletis pomonella*, is *Enterobacter agglomerans*. Several strains of this bacterium were identified from AMF, their environment, and bird feces fed on by AMF (Lauzon, 1988, 1991; MacCollom et al., 1992, 1994; Lauzon et al., 1998). One strain proved highly attractive to AMF in field tests where it performed as well as apple volatiles and increased overall attraction when it was combined with apple volatiles on Ladd traps (MacCollom et al., 1992). The same strain outperformed both ammonium acetate and another strain of *E. agglomerans* obtained from the ATCC culture collection (MacCollom et al., 1994). Another strain of *E. agglomerans* isolated from apple leaf samples taken from the habitat of AMF proved more attractive than a strain isolated from chicken feces (Lauzon et al., 1998). Finally, uricase (+) strains of both *E. agglomerans* and *E. cloacae* isolated from AMF alimentary canals were more attractive to AMF than uricase (−) strains of each species (Lauzon et al., 2000). These studies confirmed the hypothesis of Prokopy et al. (1993) that bird feces containing microorganisms could generate volatiles more attractive to AMF than feces that did not contain them, although the specific ability to metabolize uric acid to ammonia was not discussed.

Initial chemical analyses of one AMF strain attractive to the AMF (later determined uricase (+)) were carried out by Epsky et al. (1998). They showed that this uricase (+) strain was attractive to the Caribbean fruit fly (*Anastrepha suspensa*), quantified the release of ammonia and 3-methylbutanol from the bacterial cultures, and demonstrated that combinations of these two chemicals were more

attractive to the flies than either alone. Robacker and Lauzon (2002) tested attractiveness to the Mexican fruit fly (MFF) of this uricase (+) strain and a uricase (−) strain isolated from AMF. They showed that the uricase (+) strain was the more attractive and that the two strains produced qualitatively and quantitatively different volatiles as a result of metabolizing a culturing medium that contained uric acid as its principal nitrogen source.

Having demonstrated differences in attractiveness to MFF and volatiles produced by uricase (+) and uricase (−) strains of *E. agglomerans* (Robacker and Lauzon, 2002), C. R. Lauzon hypothesized that a bacterial strain isolated from a particular species of fruit fly might be more attractive to that species than a strain isolated from another fly species. To investigate this hypothesis, a uricase (+) strain of *E. agglomerans* was isolated from the MFF for comparison with the same uricase (+) AMF strain used in the work of Robacker and Lauzon (2002). Our objectives were to 1) test attractiveness of each strain to MFF; 2) identify and compare volatiles produced by the two strains; and 3) relate attractiveness of each strain with production of volatiles.

METHODS AND MATERIALS

Insects and Test Conditions. Mexican fruit flies were from a culture that originated from yellow chapote fruit (*Casimiroa greggii*, S. Wats. F. Chiang), a native citrus host of the fly, collected in Nuevo Leon, Mexico, in 1997. Mixed-sex groups of 20–25 flies (overall sex ratio 1:1, but individual cups varied) were kept in 473-ml cardboard cartons with screen tops until used in tests. Cups were provisioned with separate sugar and water sources, but no protein. Laboratory conditions for holding and testing flies were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 0630 to 1930 hr. All tests were conducted between 0900 and 1600 hr with 2–27 day-old flies. Previous work demonstrated that attraction of MFF to odors of aqueous bacterial cultures was maximum for sugar-fed, protein-deprived flies compared with other feeding regimens, and uniform between 0830 and 1630 hr under these temperature and relative humidity conditions for flies between 2–15 days post eclosion (Robacker and Garcia, 1993). However, responsiveness decreased by about 50% as flies aged from 15 to 30 days.

Bacterial Cultures and Biochemical Tests. A strain of *Enterobacter agglomerans* isolated from the midgut of wild MFF, and a strain of *E. agglomerans* isolated previously from AMF (Lauzon et al., 2000) were grown individually in tryptic soy broth (Difco Laboratories, Detroit, MI). Growth rates were monitored and compared over a 24-hr period. Growth rates were similar between the two strains, however, *E. agglomerans* cells from MFF displayed flocculent growth not observed for *E. agglomerans* from AMF. Bacterial cells were prepared for plating as described in Robacker and Lauzon (2002). Briefly, each strain was grown in nutrient medium for 18 hr at 28°C, centrifuged, and washed free of medium. Cells

were adjusted to similar optical densities (≅0.550, $\lambda = 550$ nm), plated on a uric acid medium, and sent via overnight delivery service to the USDA-ARS laboratory in Weslaco, TX for volatiles analysis and insect bioassays. After all bioassay and identification work was finished, we conducted biochemical tests using API 20E biochemical identification strips (BioMerieux, Marcy-l'Etoile, France) on both bacterial strains to determine possible reasons for changes in volatiles production that had been observed.

Wind-Tunnel Bioassay. Bioassays were conducted in a Plexiglas wind tunnel with the dimensions of $0.3 \times 0.3 \times 1.2$ m. Each end of the tunnel was screened to allow airflow. The downwind end contained a baffle system to create a uniform airflow through the chamber. Air was pulled through the chamber at 0.4 m/sec by an exhaust fan connected to the downwind end. Air exiting the chamber was directed into an exhaust hose and removed to the outdoors. In addition to the direct exhaust from the wind tunnel, this room contained inlet and outlet vents to bring in new air from the outdoors and remove air to the outdoors. Complete air replacement in the room occurred 8×/hr.

The top of the chamber had two circular openings (12.8 cm diam) with Plexiglas covers, located at each end of the chamber, to allow easy access to the interior. A 75 W "soft white" light bulb (General Electric Co., Cleveland, OH) in a reflecting lamp was positioned 17 cm above the downwind end. The purpose of this light was to minimize random flying into the upwind end by using the flies' positive phototactic reaction. Overhead lighting was provided by fluorescent "cool white" lights (F40CW, General Electric).

Attractiveness of plates containing either active cultures or uninoculated medium was assessed. Plates were incubated after arrival in Weslaco for 1–10 days at 30–32°C prior to testing. Most assays were conducted using incubations of 5–10 days because observations indicated that uric acid utilization was maximum during this period (judged by disappearance of uric acid crystals in medium). To conduct a trial, a plate bottom containing the active culture or uninoculated medium was placed on the floor of the chamber below the opening at the upwind end. One cup of flies was placed under the downwind opening. Flies were given 5 min to leave the cup, fly or walk upwind, and contact the plate. Upwind movement was scored if flies passed a point 2/3 of the distance from the release cup to the plate.

Sixteen batches of bacterial plates were tested. Each batch was divided into two groups that were bioassayed on different days. On 20 test days, test flies comprised four age/feeding status groups: young (2–5 days post eclosion), sugar-fed; young, sugar-deprived (for 1 day); old (12–27 days), sugar-fed; and old, sugar-deprived. Each plate type (AMF strain, MFF strain, uninoculated control) was tested equally with the four age/feeding status groups. Five–six-day-old, sugar-fed flies were tested on 8 day, 5–6 day-old, sugar-deprived (1 day deprivation) flies were tested on 2 day, and 19–27 day-old, sugar-fed and sugar-deprived flies were tested on 2 day.

Quantification of Volatiles from Cultures. Volatiles in the headspace above active bacterial cultures and above uninoculated medium were quantified by gas chromatography (GC). Plates used in these analyses were selected from every batch of inoculations that were used for bioassays. Incubation times ranged from 1 to 10 days at 30–32°C. Volatiles were sampled using solid phase microextraction (SPME) with a polydimethylsiloxane (PDMS) coated fiber (100 μm coating) (Supelco, Inc., Bellefonte, PA). The fiber was inserted into the headspace through a small hole drilled into the side of each plate just prior to volatiles collection. The hole was only slightly larger than the fiber sheath to minimize escape of volatiles. Sampling time was 1 hr at 30–32°C. On-column injection of volatiles was by thermal desorption from the SPME fiber at 220°C in a 10 cm retention gap (0.53 mm ID deactivated fused-silica) connected to the analytical column by a GlasSeal™ connector (Supelco). The analytical column was a DB-1 (60 m, 0.32 mm ID, 5 μm film) (J & W Scientific, Folsom, CA). Column oven temperature was 50°C for 5 min, then programmed at 5°C/min to 200°C. Carrier gas was helium at a linear velocity of 40 cm/sec. Analyses were conducted with a Shimadzu GC-17A (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with both flame ionization (FID) and flame thermionic (Model FTD-17) detectors. Detection was by FID for most plates, to analyze ammonia and organic chemicals that did not contain nitrogen. Detection was by FTD for others, to analyze organic chemicals containing nitrogen. GC peak areas were measured by using Millennium 2010 Chromatography Manager software (Waters Corporation, Milford, MA).

Peak areas were converted to headspace concentrations in the Petri plates using K values of the chemical analytes on PDMS fibers (Bartelt, 1997). K is defined as the amount of chemical on the PDMS fiber at the end of sampling divided by the concentration in the container at the end of sampling when the system is at equilibrium. K values at 25°C for several of the volatile chemicals identified in this study were published by Bartelt (1997), along with an equation for converting them into K values at 30°C, the incubation temperature used in this study. It was necessary to determine K values for ammonia, indole, 2-undecanone, and 2-tridecanone, also. For 2-undecanone and 2-tridecanone, K values were calculated using their Kovats indices and correction factors provided in Bartelt (1997).

For determinations of K for ammonia and indole, SPME analyses were conducted in 0.5 and 1 liter round-bottom flasks containing various amounts of the chemicals introduced in methanol solutions at 30°C. Flasks were capped with parafilm, and the air was stirred with a magnetic stir bar for 0.5 hr before the PDMS fiber was inserted through the parafilm and volatiles were collected for 1 hr. FID areas were measured using GC methods described above. Calibration curves for the two chemicals were made by syringe injections of methanolic solutions into a splitless injector. Other GC conditions were the same as for the SPME analyses. The on-column injector was not used for the solvent injections because

baseline noise prevented reliable measurement of small peaks like those produced by the ammonia standards. A comparison of FID areas obtained by splitless injection versus on-column injections was made by using higher concentrations of ammonia, indole, and several other chemicals. On-column injections yielded peak areas approximately 4× greater than splitless injections. This factor was used to correct FID values obtained by splitless injection before using them to produce calibration curves. Calibration curves were used to determine amounts of ammonia and indole on the PDMS fiber after sampling in the round bottom flasks, and these values were used to calculate K values of ammonia and indole. K values determined in this way were similar to those calculated using the equation in Bartelt (1997) where it was assumed that ammonia contains an amino group and that indole has a pyridine-like functionality.

For all chemicals, amounts on the fiber at the end of sampling Petri plates containing bacterial cultures were determined using calibration curves as described above in which syringe injections of standards were made into a splitless injector, but response factors were adjusted by 4× to quantify SPME injections of plate samplings in the on-column injector. Headspace concentrations at the end of sampling were calculated as the amount on the fiber divided by K (units of K are ml). The amount of each chemical in the headspace before sampling was calculated as the product of the concentration after sampling multiplied by 30 ml (approx. headspace volume of Petri plates), then adding the amount on the fiber at the end of sampling. The concentrations in the Petri plates before SPME sampling were calculated as the quotient of the amount in the headspace before sampling divided by 30 ml.

Identification of Volatiles Produced by Bacteria. Volatiles produced by bacteria were identified by gas chromatography/mass spectrometry (GC–MS). The method described above in which volatiles were sampled from plates using SPME PDMS was used for GC–MS analysis of three plates each of the AMF and MFF strains. The fiber was exposed inside the plates for 1 or 24 hr. Twenty-four hour samplings were done to collect sufficient amounts of the minor components for positive identification. For injection, chemicals were thermally desorbed from the fiber for 1 min in a split/splitless injector in the splitless mode at 250°C. The injector was purged after 1 min. The analytical column for GC–MS was the same DB-1 column described above. Linear velocity of helium carrier gas was 40 cm/sec. Column oven temperature was programmed from 50 to 230°C at 5°C/min, and holding at the final temperature for 19 min. GC–MS data were acquired using Hewlett-Packard 6890 GC (Hewlett-Packard Company, San Fernando, CA) with an HP 5973 Network Mass Selective Detector (EI) (electron energy = 70 eV) over a mass range of 40–550 amu. The system was controlled by an HPMS Chemstation. GC–MS identifications were based on computer matching of unknown spectra with those in the NIST 98 Library of Mass Spectra and Subsets (Hewlett-Packard).

Identification of Indole. GC-FTD indicated a large unknown peak containing nitrogen. GC–MS spectra yielded nearly equivalent matches with indole and two other compounds. Retention times of indole and the unknown in volatiles collected by SPME from a MFF strain plate were compared after injection onto the DB-1 column and onto a SupelcoWAX-10 capillary column (60 m, 0.32 mm ID, 0.25 μm film) (Supelco) using the conditions described above. The other two candidate chemicals were not commercially available for testing.

Acetyl chloride derivatization was also used to differentiate among the three candidate chemicals based on its reactivity with secondary amines such as indole and nonreactivity with tertiary amines such as the other two compounds. For this procedure, cells and some media were scraped from three MFF plates and extracted in 2 ml of dichloromethane. Ten drops of acetyl chloride (Sigma Chemical Co., St. Louis, MO) were added to a mixture containing 200 μl of the cell extract, 200 μl of pyridine, and 400 μl of benzene. The solution was refluxed for 1 hr at 65–70°C, then 1 μl of the reaction mixture was analyzed by GC on the DB-1 column.

Volatiles Collection on Activated Charcoal. Because of uncertainty that all important chemicals were detected by SPME, volatile collections were also conducted using activated charcoal. Previous findings indicate that some chemicals that do not bind well to SPME PDMS fiber coatings do bind well to activated charcoal (Lee et al., 1995; DeMilo et al., 1996; Robacker and Bartelt, 1997). For these analyses, an additional batch (batch 17) of AMF and MFF strain plates was prepared, and volatiles from these plates were pulled through ORBO^{TM} 100 activated charcoal tubes (Supelco, Bellefonte, PA) for 24 hr at 30–32°C with a flow rate of 400 ml/min. Tubes were extracted with 2 ml of acetone, and the extracts were concentrated to 100 μl for GC analysis using the same column and conditions that were used for SPME analyses. Five replications were conducted for each bacterial strain.

Chemical Attractiveness Bioassay. Cage-top bioassays as described in Robacker and Flath (1995) were used to test attractiveness of indole in bacterial volatiles. Indole (99%) was obtained from Fluka (Milwaukee, WI). Four quantities were tested in methanol: 10 ng, 100 ng, 1 μg, and 10 μg. The bioassay was conducted by placing two filter paper triangles (3 cm/side) containing 10 μl of indole solution and two papers containing 10 μl of methanol near the corners on top of an aluminum-screened cage (30 cm/side) containing 180–200 adult flies. Papers containing indole were positioned diagonally from each other on the cage top, as were the papers containing methanol. Papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction was solely responsible for responses. The number of flies beneath each paper was counted once each minute for 10 min following application of test chemicals.

Bioassays were conducted using flies of two feeding regimes: sugar-fed, protein-starved (from eclosion); and sugar-starved (for 1 day), protein-starved.

The four concentrations of indole were bioassayed in random order. Each concentration was assayed 24–36 times with both sugar-fed and sugar-starved flies.

Statistical Analyses. Effect of bacterial strain on headspace concentrations of volatiles was analyzed using SuperANOVA (Abacus Concepts, 1989). Analyses of variance used randomized complete block designs. Separate analyses were conducted for each chemical. Attractiveness of the two strains of bacteria in wind-tunnel bioassays was also tested by analysis of variance using SuperANOVA. For analysis, proportions of flies that moved upwind or contacted the plates in each bioassay were transformed by arcsin of the square root, because means were highly correlated with variances (Snedecor and Cochran, 1967). Proportions of 0 were replaced with $1/(4N)$. Separate analyses were conducted for males and females of different age/feeding status groupings. Means separations were done by Fisher's protected least significant difference (LSD) method in all ANOVAs.

GC area counts of several headspace chemicals from AMF strain plates that produced indole were compared with counts from AMF strain plates that did not produce indole by using *t* tests. Attractiveness of AMF strain plates from batches that produced indole was also compared with that of AMF strain plates that did not by using *t* tests.

Linear regression (SuperANOVA) was used to relate attractiveness of plates with headspace concentrations (FID area counts) of chemicals in plates. For these analyses, attractiveness indices were calculated for both males and females that responded to each bacterial strain on each test day. For example, the attractiveness index for males responding to AMF strain plates on a particular test day was calculated as follows: (the total males that flew upwind toward AMF strain plates + 3× the total males that contacted the plates)/the total males tested that day with AMF strain plates.

Paired *t* tests were used to analyze results of cage-top bioassays that evaluated attractiveness of indole. For paired *t* tests, the total of 10 counts of flies at filter papers containing test chemicals were compared with the total at papers containing solvent for each bioassay.

RESULTS AND DISCUSSION

Chemical Identifications and Quantifications. GC-FID and GC-FTD analyses of SPME volatiles collections revealed about 100 chemicals in the headspace of the bacterial plates. All chemicals considered important in previous work with the AMF uricase (+) strain were found. No peaks with areas greater than 1 mV were found by using activated charcoal that were not found by using SPME. Thus, no peaks found by using activated charcoal were quantified.

Chemicals considered important in previous work (Robacker and Lauzon, 2002) were requantified. Additional peaks were considered if the following three conditions were met: 1) peak areas were at least 1 mV; 2) peaks were found

in at least three different volatiles samplings; and 3) peaks were found in significantly greater amounts in at least one of the two strains than in the uninoculated controls. Chemicals identified by GC–MS that met these criteria were 3-hydroxybutanone, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, 2-undecanone, and 2-tridecanone. Ammonia, identified by GC-FID only, also was quantified. Four additional chemicals met the criteria, but could not be identified by GC–MS either because of nonunique spectra or low peak areas. No attempt was made to identify three of the unknowns that had peak areas just above the 1 mv threshold.

The other unknown yielded large peaks and contained nitrogen as indicated by FTD/FID peak area ratios (8:1). GC–MS indicated a molecular weight of 117.06 and molecular formula of C_8H_7N, but could not distinguish among indole, 5H-1-pyridine, and indoline. Retention times of the unknown on DB-1 and SupelcoWAX-10 columns matched those of indole. Extracts of MFF strain culture treated with acetyl chloride showed a 100% reduction in GC peak area of the unknown compound. Reflux of controls without acetyl chloride showed no reduction in the unknown compound. This demonstrated that the unknown was a primary or secondary amine. As indole is a secondary amine and 5H-1-pyridine and indoline are tertiary amines that do not react with acetyl chloride, this indicated the unknown was indole.

GC-FID quantifications of headspace chemicals are shown in Table 1. Data are headspace concentrations calculated using the method of Bartelt (1997). These were calculated because they represent amounts of the chemicals in the headspace better than do peak areas. However, these concentrations should be viewed only as approximations due to many assumptions that were made, and because concentrations vary with cell count, incubation time and temperature, culturing medium variability, and other factors.

Both bacteria strains produced more ammonia, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole than uninoculated controls. In fact, no 3-methylbutanol, 2-phenylethanol, or indole was found in uninoculated controls. The MFF strain produced more 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole than the AMF strain. However, the AMF strain produced 3-hydroxybutanone, a chemical that was not found in headspace of either the MFF strain or uninoculated controls. All of these chemicals except indole had been identified previously from bacterial odors attractive to fruit flies (Gow, 1954; Drew and Fay, 1988; Lee et al., 1995; Robacker and Flath, 1995; DeMilo et al., 1996; Robacker and Bartelt, 1997; Epsky et al., 1998; Robacker et al., 1998; Robacker and Lauzon, 2002). Indole has been reported from *E. agglomerans* (11–25% of strains) and other Enterobacteriaceae (Brenner, 1984; Yu et al., 2000). In addition, 2-undecanone and 2-tridecanone are also known from volatiles produced by *E. coli* (Yu et al., 2000), a bacterium closely related to *E. agglomerans* (Lauzon et al., 1998).

TABLE 1. CONCENTRATIONS OF CHEMICALS IN HEADSPACE OF PETRI PLATE CULTURES OF TWO STRAINS OF *E. agglomerans*

Bacteria strain	*N*	NH3	3HyB	3MeB	25DMP	2PhEt	Indole
Uninoculated medium[a]	15	2.7a (±0.9)	0.0a (±0.0)	0.0a (±0.0)	0.07a (±0.02)	0.0a (±0.0)	0.0a (±0.0)
AMF strain[a]	15	330b (±160)	6.8b (±1.1)	47b (±10)	0.22b (±0.04)	0.33b (±0.07)	4.4b (±1.8)
With indole[b]	6	220 (±71)	5.8 (±2.1)	77* (±19)	0.31 (±0.07)	0.45 (±0.08)	11* (±3.2)
Without indole[b]	9	400 (±250)	7.4 (±1.2)	27* (±3.6)	0.16 (±0.04)	0.24 (±0.09)	0.0* (±0.0)
MFF strain[a]	15	220b (±59)	0.0a (±0.0)	94c (±14)	0.36c (±0.05)	0.55c (±0.07)	8.7c (±1.2)

[a] Mean concentrations (ng/ml) (±SE) for a chemical followed by the same letter were not significantly different from each other by Fisher's protected LSD ($P < 0.05$) based on ANOVAs of FID peak areas measured from each GC determination. Standard errors denote sampling error from determination of FID peak areas; they do not estimate error from determination of concentrations from peak areas.

[b] Significant differences in headspace concentrations between replications of AMF strain plates that produced indole vs. replications that did not produce indole were determined using *t*-tests and are indicated by an asterisk (*).

Of the 16 batches of plates prepared over approximately 1-year duration of this work, nine of the AMF strain batches produced indole and six did not (Table 1). Batches of plates that produced indole were not randomly distributed over time but clumped. Batches 1–2 did not produce indole, batches 3–6 did, batches 8–13 did not (batch 7 was not tested by GC), batches 14–15 did, and batch 16 did not. All of the MFF strain plates produced indole. For batches in which AMF strain plates produced indole, headspace concentrations of indole in AMF strain plates were positively correlated with indole concentrations in MFF strain plates ($r = 0.96$, $df = 8$, $P < 0.001$).

Data in Table 1 were partitioned to compare headspace concentrations of various chemicals for AMF strain plates that produced indole versus those that did not. AMF plates that produced indole also produced other chemicals, except 3-hydroxybutanone, in amounts similar to those produced by the MFF plates. This was most notable for 3-methylbutanol that was found in significantly higher concentrations in AMF plates that produced indole compared with those that did not. 3-Hydroxybutanone was produced nearly equally in AMF plates that produced versus did not produce indole.

2-Undecanone and 2-tridecanone were not included in Table 1 because their headspace concentrations were very low, and no simple ketones are known to be attractive to MFF. The headspace concentrations of 2-undecanone were 33 ± 11 (SE) and 79 ± 21 pg/ml in AMF strain and MFF strain plates, respectively. The headspace concentrations of 2-tridecanone were 18 ± 3.5 and 21 ± 6.1 pg/ml in AMF strain and MFF strain plates, respectively.

Generally, concentrations of chemicals were comparable to those reported in previous studies of volatiles produced by bacteria attractive to the MFF (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al., 2000). However, the concentration of ammonia was lower than that found in most previous work and notably was lower than that reported in our previous work with the same AMF uricase (+) strain of *E. agglomerans* (Robacker and Lauzon, 2002).

Attractiveness of AMF and MFF Strains. On the 20 test days in which four age/feeding status groups of flies were bioassayed, older females flew upwind toward ($F = 10.4$; $df = 1,195$; $P < 0.01$) and landed on ($F = 9.7$; $df = 1,195$; $P < 0.01$) plates (three plate types combined) more often than younger females. Age did not affect male responses. Hunger status had no effects on either males or females. Interactions of age and hunger status with bacterial strain (AMF vs. MFF strains or replications of the AMF strain that produced indole vs. those that did not) were not significant indicating that fly age and hunger status did not affect responses to the different strains. Therefore, all bioassay results were combined for further analysis without regard to fly age or sugar-feeding regime.

Summed over all bioassays, more males and females flew upwind toward and landed on AMF strain plates than on either uninoculated or MFF strain plates ($F = 14.8$, 12.7, 22.9, 17.5 for males upwind, females upwind, males landing,

TABLE 2. ATTRACTION OF MEXICAN FRUIT FLIES TO PETRI PLATES CONTAINING TWO STRAINS *E. agglomerans* OF IN A WIND TUNNEL

	Bacteria strain	*N*	Upwind movement	Contact with plate
Males	Uninoculated medium[a]	101	11.4 ± 1.0a	3.2 ± 0.5a
	AMF strain[a]	109	18.7 ± 1.3b	7.2 ± 0.8b
	With indole[b]	35	14.9 ± 1.9*	3.9 ± 1.0*
	Without indole[b]	67	21.1 ± 1.8*	9.1 ± 1.1*
	MFF strain[a]	109	11.5 ± 1.0a	1.8 ± 0.4a
Females	Uninoculated medium[a]	101	11.0 ± 1.0a	2.6 ± 0.6a
	AMF strain[a]	109	17.7 ± 1.3b	6.9 ± 0.8b
	With indole[b]	35	18.9 ± 2.4	6.8 ± 1.6
	Without indole[b]	67	17.3 ± 1.6	7.0 ± 1.0
	MFF strain[a]	109	11.0 ± 1.0a	2.3 ± 0.5a

[a] Values are mean percentages (±SE) responding out of the total flies in the trial. N = trials per treatment. Means in the same sex and same column followed by different letters are significantly different at the 5% level by Fisher's protected LSD.

[b] Significant differences in responses between replications of AMF strain plates that produced indole vs. replications that did not produce indole were determined using t-tests and are indicated by an asterisk (*).

females landing, respectively; $df = 2,301$; $P < 0.001$) (Table 2). Responses to the MFF strain were not significantly different from responses to uninoculated controls using the complete model. However, fewer males landed on MFF strain plates than on uninoculated plates using a model that did not include AMF strain plates ($t = 2.3$, $df = 193$; $P < 0.05$). Among AMF strain plates, those that produced indole were less attractive to males than those that did not (upwind movements: $t = 2.2$, $df = 100$; $P < 0.05$; landings: $t = 3.1$, $df = 100$; $P < 0.01$). Effects of indole on responses of females were not evident.

Bioassays of Indole. Attractiveness of indole in cage-top bioassays was significant by paired t tests ($P < 0.05$) for starved flies at 10 ng, 100 ng, and 10 μg test quantities. Differences between counts at filter papers containing indole and control papers with solvent (methanol) were positive for all test quantities, and no dose–response relationship was evident so data were combined over test quantities resulting in mean counts (per bioassay) of flies at papers containing indole of 35.2 ± 1.0 (SE) ($N = 120$ bioassays) and mean counts at solvent papers of 29.3 ± 1.0. The difference was significant by a paired t test ($t = 5.2$, $df = 118$, $P < 0.01$), indicating that indole was attractive to sugar-starved flies.

Indole was not significantly attractive to sugar-fed flies at any individual test quantity although the difference between counts at papers containing indole and solvent papers was positive at the lowest three test quantities (10 ng, 100 ng, 1 μg). Assuming that the highest test quantity (10 μg) was too high to be attractive, data were combined over the three lowest test quantities for analysis. For this combination, mean counts (per bioassay) of flies at papers containing indole

were 16.6 ± 0.9 (SE) ($N = 84$ bioassays) and mean counts at solvent papers were 14.0 ± 1.0. The difference was significant by a paired t test ($t = 2.4$, $df = 82$, $P < 0.05$), indicating that indole was attractive to sugar-fed flies.

These results show that indole is somewhat attractive to MFF. For sugar-starved flies, counts at indole papers were only 20% greater than counts at solvent papers. For sugar-fed flies, counts at indole papers (three lowest test quantities) were only 19% greater than counts at solvent papers. These results suggest that indole probably would not play a major role in attractiveness of the bacterial plates.

Lack of Attractiveness of MFF Strain Plates. MFF strain plates produced more ammonia, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole, than uninoculated plates. However, MFF strain plates were not more attractive than uninoculated plates to females, and were less attractive to males (Table 2, discussion above). As we found no other chemicals produced by the two plate types that differed significantly in emissions, the most prudent conclusion is that one or more of the chemicals produced by the MFF strain plates inhibited attraction.

To determine if chemicals produced by MFF strain plates inhibited attractiveness, we analyzed linear regression of attractiveness indices (% of flies to move upwind + 3×% of flies to contact the plates, per test day) versus headspace concentrations of ammonia, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole in MFF strain plates. No models with one or more of the chemicals were significant for either male or female indices. We considered that lack of significant correlations did not invalidate the inhibition hypothesis because MFF strain plates were not actually attractive, such that whatever apparent differences occurred in attractiveness indices were probably due to random variation.

We reasoned that if inhibition by one or more chemicals could be shown in the AMF plates, then the same effect could account for the low attractiveness of the MFF plates. For AMF strain plates, the linear regression of attractiveness indices of males versus indole was significant ($r = -0.52$, $df = 23$, $P < 0.01$). Recall also that fewer males moved upwind toward and contacted AMF strain plates that produced indole compared with plates that did not produce indole (Table 2). No other models with one or more of the other four chemicals were significant for male attractiveness indices. No models containing one or more of these five chemicals were significant for female attractiveness indices. The best fit was a negative correlation between attractiveness index and headspace concentration of indole ($r = -0.33$, $df = 23$, $P = 0.1$). These results suggest that indole may have inhibited attraction to the AMF strain plates.

Assuming that the five chemicals under consideration were the only ones affecting attractiveness of plates, we combined data for AMF and MFF strain plates. Negative correlations of indole concentration with attractiveness indices were significant for both males and females (males: $r = -0.52$, $df = 48$, $P < 0.001$; females: $r = -0.34$, $df = 48$, $P < 0.05$). Negative correlations were also significant for 2,5-dimethylpyrazine (males: $r = -0.36$, $df = 28$, $P = 0.05$; females:

$r = -0.49$, $df = 28$, $P < 0.01$) and 2-phenylethanol (males: $r = -0.44$, $df = 28$, $P < 0.05$; females: $r = -0.45$, $df = 28$, $P < 0.05$). Multiple regression models did not account for significantly greater variation in attractiveness index for either males or females. Headspace concentrations of indole were positively correlated (but not significant at the 5% level) with concentrations of 3-methylbutanol, 2,5-dimethylpyrazine, and 2-phenylethanol. Further, headspace concentrations of 3-methylbutanol, 2-phenylethanol, and 2,5-dimethylpyrazine were positively correlated with each other (smallest $r = 0.64$, $df = 13$, $P = 0.05$). These results suggest that several of these chemicals may have acted together to inhibit attractiveness.

Bioassays of indole indicated that it was slightly attractive to both sugar-fed and sugar-starved flies. Also, ammonia (Robacker et al., 2000), 2,5-dimethylpyrazine (Robacker and Warfield, 1993; Robacker and Flath, 1995), and 2-phenylethanol (Robacker and Lauzon, 2002) are all known to be attractive to MFF. These results apparently contradict the analyses showing negative correlations of one or more of these chemicals with attractiveness.

However, there is precedence for attractive chemicals inhibiting other attractive chemicals in the MFF. As examples, pyrrolidine (Robacker and Warfield, 1993), dimethylamine, ethylamine, and 2,5-dimethylpyrazine (Robacker et al., 1997), acetic acid (Robacker et al., 1996), and a mixture of 1,8-cineole, hexanol, ethyl hexanoate, and ethyl octanoate (Robacker and Heath, 1997), each inhibited attraction of MFF to various attractive mixtures containing ammonia and other amino compounds, even though each of these inhibitory chemicals was attractive when tested alone. Also, chemicals that are attractive at low concentrations can become repellent at higher ones. Examples of this effect are putrescine (Robacker and Warfield, 1993) and ammonia (Robacker et al., 1997) that each enhanced attractiveness in mixtures containing them and methylamine. Each, however, also depressed attractiveness if their concentrations were raised to levels that actually were most attractive when each chemical was tested by itself. There was a suggestion that indole may have enhanced attractiveness of AMF strain plates to females, as its headspace concentration increased from 0 (attractiveness index = 0.4) to about 5 ng/ml (index = 0.7), then depressed attractiveness as its concentration increased to about 20 ng/ml (index = 0.1), but the effects were not significant.

Attractiveness of AMF Strain Plates. AMF strain plates were significantly more attractive than MFF strain plates even though MFF strain plates had higher concentrations of 2,5-dimethylpyrazine, 2-phenylethanol, and indole. AMF strain plates, but not MFF strain plates, produced 3-hydroxybutanone, another chemical attractive to this fly (Robacker and Lauzon, 2002). Concentrations of ammonia in the headspace of the two strains were not significantly different.

Considering that headspace of each plate type contained the same chemicals except 3-hydroxybutanone, two explanations for these results can be hypothesized: 1) higher concentrations of some of the attractive chemicals in the MFF plates inhibited attraction relative to the AMF strain plates; and/or 2) 3-hydroxybutanone

increased attractiveness of AMF strain plates. The first explanation was verified as a possibility in regression analyses that showed negative correlations of attractiveness with 2,5-dimethylpyrazine, 2-phenylethanol, and indole.

The hypothesis that 3-hydroxybutanone was responsible for the greater attractiveness of AMF strain plates was examined by linear regression of attractiveness indices versus headspace concentration of 3-hydroxybutanone in AMF strain plates. Although the purpose was to determine why AMF strain plates were more attractive than MFF strain plates, the initial analyses were done using only AMF strain plates because MFF strain plates did not produce 3-hydroxybutanone.

Correlations of attractiveness indices of AMF strain plates versus headspace concentration of 3-hydroxybutanone were positive, but not significant, for both males and females. Lack of significance does not invalidate the hypothesis, but indicates that differences in attractiveness of AMF plates from each other cannot be attributed unequivocally to differences in concentrations of 3-hydroxybutanone. When results for MFF strain plates and AMF strain plates were combined, the correlation was significant for both males and females (males: $r = 0.44$, $df = 28$ $P < 0.05$; females: $r = 0.51$, $df = 28$, $P < 0.01$). In this case, differences in concentrations of 3-hydroxybutanone are consistent with differences in attractiveness, but do not prove that 3-hydroxybutanone is responsible for the greater attractiveness of AMF strain plates compared with MFF strain plates.

Changes in the AMF Strain. We presented evidence that the AMF strain changed during the course of this work, at times producing volatiles that were considerably different from those produced by the MFF strain plates and at other times producing indole and other volatiles similar to those produced by MFF strain plates. Indications that attractiveness also changed as the volatiles changed were evident in responses of males (Table 2). We did not observe consistent changes in volatiles production or attractiveness of plates with length of incubation time from 1–9 days.

In addition to changes that occurred during the current work, we also observed differences in AMF strain plates compared to previous work with the same uricase (+) strain of *E. agglomerans* (Robacker and Lauzon, 2002). In our former work, attraction to AMF strain plates was about 8× greater than attraction to uninoculated plates, whereas in the present study, the ratio was only about 2×. Indole was not found in our previous work. We were unable to determine if amounts of any other chemicals were different from our previous work.

To attempt to understand variation of volatiles profiles, we examined more extensively the biochemical capabilities of our strains of *E. agglomerans*. We found that subpopulations (cultured and plated as described in the Methods section), hereafter referred to as minority or majority subpopulations (or phenotypes), existed within both AMF and MFF strains. These minority subpopulations were different in terms of their ability to utilize substrates, and their colonial morphologies. The AMF strain contained one majority and one minority phenotype. The MFF strain contained one majority and two minority phenotypes.

The majority AMF subpopulation completely utilized uric acid when grown on the uric acid medium. The medium maintained a peach color indicating that the medium was near neutral pH. This indicates that utilization of the uric acid was likely accompanied by production of acidic fermentation products. This is substantiated in part by our chemical results. The color of the medium remained constant for approximately 7 days, and then the medium became progressively more alkaline (pink) as the remaining uric acid was utilized and other constituents within the medium were spent.

The minority AMF subpopulation did not completely utilize uric acid when grown on uric acid medium under identical conditions and time. Instead, the subpopulation demonstrated only partial utilization of the uric acid; the medium was more alkaline and was pink in color. Acid by-products were either absent or in amounts that did not affect the pH of the medium enough to detect them visually.

The majority MFF subpopulation gave observable traits on the uric acid medium similar to those seen for the minority AMF subpopulation. The majority MFF subpopulation produced compounds that turned the UA medium a deep, bright pink color, and only partially degraded the uric acid. Therefore, acidic compounds, if produced, did not drive the pH of the medium down. This may be explained by the fact that the majority MFF subpopulation did not follow a butylene glycol fermentation route from pyruvate. The majority AMF subpopulation, however, did and likely produced acidic compounds that appreciably altered the pH of the medium toward acidity. This explains why the AMF strain produced 3-hydroxybutanone and this compound was not detected for the MFF strain. 3-Hydroxybutanone is an intermediate of butylene glycol fermentation.

The majority MFF subpopulation and the minority AMF subpopulation also produced indole from tryptophan degradation. The majority AMF subpopulation did not. The emergence of a minority subpopulation within the AMF strain that produces indole may explain why during our previous work the AMF strain was more attractive to MFF than during our current studies.

Two minority subpopulations were isolated from the majority MFF subpopulation. The majority colonial morphology resembled the majority AMF subpopulation morphology with one exception. The majority MFF subpopulation was rougher in colonial texture than the typical smooth colonies of the AMF subpopulation. Both colonial types were circular to irregular, translucent, and pigmented, pale-yellow. The minority colonial morphologies included a smooth yellow colony and a smooth, off-white colony. All three MFF subpopulations grown individually on uric acid medium gave different observable results. As mentioned earlier, the majority MFF subpopulation cleared the uric acid to some extent, and the medium turned deep pink in color. The smooth yellow MFF subpopulation utilized uric acid less than the majority subpopulation, and the medium turned to a color close to that of peach, a combination of yellow, orange, and pink. This reaction was

most similar to that associated with the majority AMF subpopulation. The smooth off-white colony did not appear to utilize uric acid, and the medium turned yellow. Equal combinations of all three subpopulations yielded partial utilization of uric acid and a yellow medium. Combination of the two minority subpopulations resulted in a peach-colored plate. Therefore, despite the fact that the majority MFF subpopulation alone gave a strong alkaline reaction on the UA plate, in mixed company, the end result was quite different.

These findings suggest that microbial population ecology is an important aspect of chemical ecology and insect behavior. The population shifts that we observed may have been a result of repeated subculture within the laboratory setting or may reflect polymorphisms specific to each fruit fly species. *Enterobacter agglomerans* has been isolated routinely from fruit flies in four genera of economically important Tephritidae, their host fruit, oviposition sites, and in their natural food. These commonalities are intriguing and additional biochemical and molecular examination of strains of *E. agglomerans* may provide important insight toward insect behavior and biology.

Effect of Uricase on Volatiles and Attractiveness. The title of a previous paper (Robacker and Lauzon, 2002) implied that the ability of *E. agglomerans* to metabolize uric acid affected volatiles production and attractiveness to MFF, although these conclusions were not stated in the paper. These ideas were based on findings that a uricase (+) strain, but not a uricase (−) strain, produced 3-hydroxybutanone, that the uricase (+) strain produced greater amounts of several other volatiles compared with the uricase (−) strain, and the uricase (+) strain was more attractive than the uricase (−) strain. However, in the current research, we showed that two uricase (+) strains differed just as much in volatiles production and attractiveness as did the uricase (+) and (−) strains in the previous work. Taking results of both studies into account, there is no reason to conclude that differences in volatiles production and attractiveness are tied to whether or not the strains can metabolize uric acid.

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